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Antibody Specificity to Oncogene-Derived Peptides for Breast Cancer Detection

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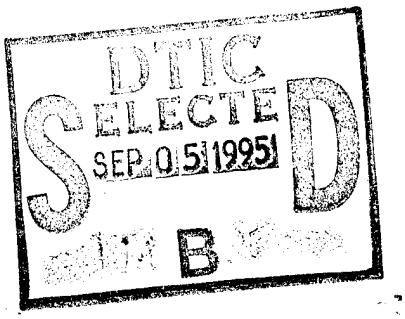

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This report describes the initial work carried out on the specifcity of two peptide-antibody interactions. Monoclonal antibody 172-12A4, which was raised against the peptide LGSGAFGTIYKGC from *v-erbB*, was screened against several peptide libraries and yielded individual sequences that were recognized with high affinities. Also, a complete set of individual substitution analogs for each position of the antigenic determinant of this peptide was synthesized. This set of analogs was assayed by direct and competitive ELISA to determine the replaceability and relative positional importance of each residue. Four out of eight residues were found to be highly specific in which no substitution was permitted. In the second example, mAb 121-15B10, which was raised against IGRGNFGEVFSGC from *v-fes*, was screened against peptide libraries yielding a profile of specific and redundant residues. The understanding of the specificities of these antibodies at the amino acid level will help in the development of these antibodies as early diagnostics in breast cancer.

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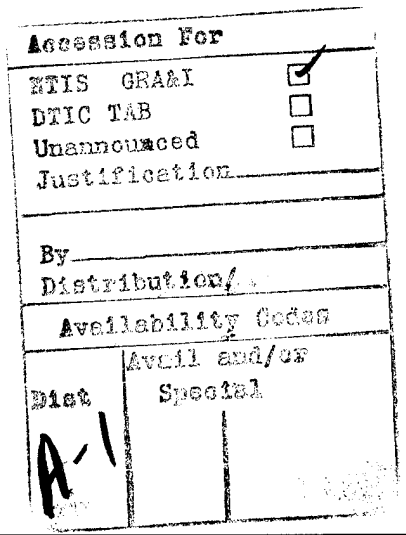

Clemencia Pinilla

PI - Signature Date

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INTRODUCTION

It has been shown that many proteins encoded by oncogenes (*c-erbB-2)* and growth factor receptors (EGFR) are implicated in breast cancer. These proteins contain a consensus region that has homology with the catalytic core domain of protein kinases. The presence of these proteins involved in cell regulation and growth in clinical samples of breast cancer patients has initiated numerous studies toward the development of antibody based-detection methods for early and effective diagnosis and prognosis of breast cancer. Peptide-specific monoclonal antibodies have also been shown to be able to detect these proteins in patient serum and/or urine samples, thus offering the potential for the ready detection and monitoring of the progression of the disease.

This study will focus on the elucidation of the specificities of a number of monoclonal antibodies raised against four different synthetic peptides derived from homologous regions of oncogene proteins. The potential use of these antibodies as specific probes to detect breast cancer-related protein markers in patient serum samples is under study (collaboration with Dr. Niman). To further develop detection strategies using these antibodies, it is essential that their binding characteristics be determined at the amino acid level. The specificity at the amino acid level of these peptide-specific antibodies can be readily determined using complete sets of substitution analogs prepared by the method of simultaneous multiple peptide synthesis (SMPS) (1). When assayed against monoclonal antibodies by competitive ELISA, sets of substitution analogs provide a relative rank order of importance for each of the individual amino acids being substituted at a given position of an antigenic determinant and also for each position making up the antigenic determinant (2,3).

Peptide libraries composed of millions of peptide sequences offer a unique advantage to fully understand the recognition capabilities of a monoclonal antibody for different peptide sequences. Synthetic peptide combinatorial libraries (SPCLs), codeveloped by the principal investigator, offer the advantage of using nonsupport-bound peptides in relevant quantities needed for solution assays (first described in 4 and 5, and reviewed in 6). In this manner, we will determine the extent of crossreactivity of these antibodies for other oncogene-derived proteins and funtionally-related proteins that share these sequences with protein kinases.

The antibody recognition patterns derived from this study will further our understanding of these specific interactions at the amino acid level, and will offer new approaches, such as using combinations of monoclonal antibodies, for the identification of protein products derived from oncogenes responsible for breast cancer. The use of well-characterized monoclonal antibodies will lead to earlier and more precise detection of relevant proteins in patient samples. These antibodies can also be used to monitor the progression of the disease in those cases where intervention and treatment may be necessary.

This report describes the initial results on the study of two different peptide-antibody interactions. Monoclonal antibodies were assayed by ELISA against substitution analogs and peptide libraries and yielded information regarding the

detailed specificities of these interactions at the amino acid level. A portion of this work was recently presented (7).

BODY

Peptide synthesis

Individual substitution analogs of the peptide LGSGAFGTIYKGC were prepared by SMPS (1). Each position of the antigenic determinant (AFGTIYKG) was replaced with one of the other 19 L-amino acids, yielding 152 peptides. Peptide purity was analyzed by RP-HPLC for each analog and ranged between 65-95%. Peptides were assayed by ELISA without any further purification. Mass spectral analysis confirmed the expected mass for several control peptides from the synthesis. Peptide controls were prepared in a similar manner.

Peptide libraries

A number of peptide libraries were screened against each monoclonal antibody, including the dual-defined libraries, hexa- and decapeptide positional scanning libraries, length libraries, and two different sublibraries. These sublibraries were prepared specifically for mAb 172-12A4 and were designed to reduce the diversity and maximize the signals of the most active peptide mixtures from the initial library screening. This in turn minimized the number of individual peptides ultimately prepared.

Direct ELISA

Direct ELISA was used to assay the substitution analogs for antibody recognition (2,3). Peptides were adsorbed to microtiter plates at concentrations of 0.2μM and 2μM per well by incubation in 0.06M sodium bicarbonate/0.03M sodium carbonate buffer, pH 9.3, overnight at room temperature in a moist box. The plates were washed 10 times with deionized water to remove unbound peptide. Nonspecific absorption of antibody was blocked by incubating the plates with a 100μl of 1% bovine serum albumin (BSA) (Sigma) in phosphate buffered saline (PBS) for 1 hour at 37° C. Monoclonal antibody was diluted with 1% BSA/PBS to give a maximum optical density (O.D.) reading for the original peptide, serially diluted down the plate, and then incubated at 37° C for 1 hr or 4°C overnight. Plates were washed as before. Horseradish peroxidase-conjugated goat antimouse IgG (Calbiochem) diluted with 1% BSA/PBS to 1:5000 50μl/well was added and the plates were incubated at 37° C for 1 hour. Excess antibody-conjugate is removed by washing as before. The amount of antibody-conjugate bound in each well was quantitated by reaction for 10 minutes with 50μl/well of freshly prepared developing solution consisting of o-phenylenediamine (Sigma) and 25μl of 3% hydrogen peroxide in 6ml of deionized water. The enzymatic reaction was stopped with 25μl/well of 4N sulfuric acid, and the resulting color was read at 492nm by a Titertek Multiscan spectrophotometer. The recognition of each

peptide analog is expressed as percentage of the antibody binding to the original peptide. Each peptide analog was assayed a minimum of three times.

Competitive ELISA

Competitive ELISA permits a more accurate quantification of the relative binding of peptide analogs to antibody and lessens undesirable effects caused by differential binding of individual peptide analogs to the ELISA plate (3). Also, peptide libraries were screened by competitive ELISA. Competitive ELISA is carried out in a similar manner as the direct ELISA described above, with the following modifications: 1) only the control peptide is bound to the plate at an optimal concentration; and 2) serial concentrations of each peptide analog or peptide mixture are added before the addition of an optimal concentration of specific antibody. The concentration of each analog or peptide mixture necessary to inhibit 50% of the binding of the antibody to the original peptide on the plate was determined (IC_{50}) using Graphpad (ISI Software). To determine the relative replaceability of each position of the antigenic determinant from the substitution analog results, the IC_{50} of each peptide analog was divided by the IC_{50} of the control peptide to yield the replaceability factor (RF) for each peptide analog. Individual RFs are a relative measure of each analog's ability to inhibit mAb as compared to the control peptide, which has an RF of 1.0. Thus, an analog with an RF less than 1.0 is one for which a lower concentration of analog is needed to inhibit 50% of mAb binding to the control peptide, and vice versa. To determine the overall replaceability of each position within the antigenic determinant, the average of the RFs of the 19 substitution analogs at each position is termed the relative positional importance factor (RPIF). This number represents the relative functional contribution to antibody binding of each position within the antigenic determinant.

LGSGAFGTIYKGC/mAb 172-12A4

Substitution analog profile

Direct ELISA data in Figure 1 is represented as the percent of MAb 172-12A4 bound to each of the 152 analogs relative to the control peptide LGSGAFGTIYKGC, from residues 138-149 of *v-erbB*. The substitution profile revealed that mAb 172-12A4 recognized a discontinuous linear determinant, in which four residues (F, G, I, K) were specific and four residues were relatively redundant. Interestingly, glycine appeared twice in the antigenic determinant, each playing a different role. At postion 3 of the antigenic determinant, glycine was highly specific, but the same amino acid at position 8 was redundant. This difference in specificity was also found for phenylalanine at position 2 (specific) and tyrosine, which is often a conservative replacement of phenylalanine, at position 6 (redundant).

Competitive ELISA data is shown as the relative positional importance factor (RPIF). The concentration of each substitution analog necessary to inhibit 50% (IC_{50}) of MAb 172-12A4 binding to the control peptide adsorbed to the plate was determined. IC_{50} values for the 19 analogs at each position were averaged. This averaged IC_{50}

value is termed the relative positional importance factor (RPIF) and represents the overall replaceability of a given position in the antigenic determinant. An RPIF of 1.0 indicates that there is no difference in recognition for the 19 substitution analogs relative to the control peptide (IC_{50}=200nM). A high RPIF represents a relatively specific position. It was found that mAb 172-12A4 recognized a discontinuous linear determinant, in which four residues were specific and four residues were relatively redundant. The specific residues were phenylalanine (RPIF=40.4), the next residue glycine (RPIF=21.4), isoleucine (RPIF=5.5), and lysine (RPIF=20.3).

Positional scanning library

The screening of libraries permit the systematic study of crossreactivity, since these libraries are composed of nearly all of the possible combinations of peptides of a certain length. The positional scanning screening data yielded the most active amino acid residues at each position for a hexapeptide sequence (Figure 2). This screening data was sufficient to locate the specific residues of the antigenic determinant, namely positions 2-7 since alanine and glycine at positions 1 and 8, respectively, were redundant. Tyrosine in position 1 of the library was nearly five-fold better than the expected phenylalanine. In this case, tyrosine represents a conservative substitution of the phenylalanine residue.

At this point we prepared two sublibraries in the same positional scanning format containing only the amino acids that were found to be active from the initial library screening. Upon synthesis and screening of these two sublibraries, 72 individual peptides derived from the combinations of the most active amino acid at each position of the library were synthesized and assayed (Table 1). Peptides having proline at the third position were at least 10-fold more active than those having glycine at the same position. Also, peptides having tyrosine at the second position were poorly recognized, indicating that the peptides responsible for the activity found for Ac-XYXXXX-NH_2 are not the same as those in Ac-YXXXXX-NH_2. The most active peptides, outline in Table 1, were found to be analogs of the antigenic determinant, although their activities were lower. This fact demonstrates that a sequence longer than six residues is required for high affinity binding.

The screening profile for the decapeptide positional scanning library revealed similar results as the hexapeptide library, in which only the specific residues displayed significant inhibition (data not shown).

Length library

Another library was screened to obtain information on the optimal length for the antigens recognized by mAb 172-12A4. This library consisted of six different sets of peptide mixtures having the first position defined with one of the 20 L-amino acids and the remaining positions as mixtures. Assayed by competitive ELISA, this length library yielded the most active amino acids in the first position of the antigens recognized by mAb 172-12A4 (Figure 3). The optimal lengths appeared to be seven and eight residues, supporting the results of the hexapeptide positional scanning library.

Dual-defined library

Each of the dual-defined combinatorial libraries is composed of 400 peptide mixtures, having two positions defined and four positions as mixtures. Thus, 1200 different peptide mixtures were screened by competitive ELISA and their IC_{50} values were determined. The IC_{50} values for the best peptide mixtures from each library are shown in Table 2. This data is in agreement with the hexapeptide positional scanning library. Residues -YG- are the most active residues in positions 1 and 2, and lysine is clearly the most active residue in position 6. There is some overlap of -YG- in positions 3 and 4. However, the isoleucine, which is the expected residue in position 4 of the antigenic sequence, is found in several active peptide mixtures. The connectivity of the most active amino acids from the three different libraries will be determined by synthesizing individual peptides representing the combinations of the most active peptide mixtures.

IGRGNFGEVFSGC/mAb 121-15B10

Positional scanning library

The hexapeptide positional scanning library was screened against mAb 121-15B10, which was raised against IGRGNFGEVFSGC from residues 529-540 of *v-fes*. The screening results in Figure 4 illustrate the most active amino acids at each position of the hexapeptide. The most active amino acids were isoleucine and methionine in position 1, glycine in position 2, histidine and arginine in positions 3 and 4, aspartic acid, histidine, and arginine in position 5, and aspartic acid in position 6. (Cysteine often gives anomalous results) This led to the synthesis of 24 individual hexapeptides corresponding to the combinations of amino acids selected. The activities of these peptides are shown in Table 3, with the most active sequences outlined. Thus, it is clear that mAb 121-15B10 recognizes the N-terminal region of the antigenic sequence.

Dual-defined library

The dual-defined library Ac-XXO_3O_4XX-NH_2 was also screened against this antigen-antibody interaction. The most active peptide mixture was clearly Ac-XXRRXX-NH_2, which was in agreement with the most active peptide sequences found from the positional scanning library. An iterative synthesis and selection process was carried out for this peptide mixture and is shown in Figure 5. Each remaining mixture position was individually defined with one of the 20 L-amino acids and the resulting peptide mixtures were assayed by competitive ELISA. The most active amino acids at positons 1 (isoleucine and methionine) and 2 (glycine) are in agreement with the results found from the positional scanning library. Position 5 and 6, however, yielded different results in which tyrosine was clearly specific in the fifth position and a number of hydrophobic amino acids were active in the sixth position. Final peptides are currently being synthesized.

CONCLUSIONS

The preliminary mapping of two peptide-antibody interactions has been carried out using substitution analogs and peptide libraries. The amino acid level of specificities found for these interactions is required for the use of monoclonal antibodies in the detection of oncogene-derived products. Early detection of these products implicated in breast cancer will lead to earlier treatment of this disease.

Future experiments

The direct and competitive ELISA data for the substitution analogs for LGSGAFGTIYKGC will be finalized. Also, more peptide libraries, especially those containing D- and nonnatural amino acids, will be screened against mAb 172-12A4 for the potential identification of unrelated sequences recognized by this antibody. Individual hexapeptides representing combinations of amino acids from the dual-defined libraries will be prepared and assayed to finish the hexapeptide library data. One or more iterations may be pursued from the length and decapeptide libraries in order to identify longer sequences with high activities. The final sequences identified from the library screenings will be used to search a sequence database for homology with other protein sequences.

The substitution analogs for IGRGNFGEVFSGC will be made and tested against a number of monoclonal antibodies which were raised against this peptide. Also, more libraries will be assayed against these other monoclonal antibodies.

Initial synthesis of the other two peptides from the second objective of the proposal has been carried out. ELISA experiments have begun to optimize the library screening conditions for both systems. A number of peptide libraries will then be screened against these two antigen-antibody systems. Substitution analogs will be prepared for the other two peptide-antibody systems in the next 6-12 months.

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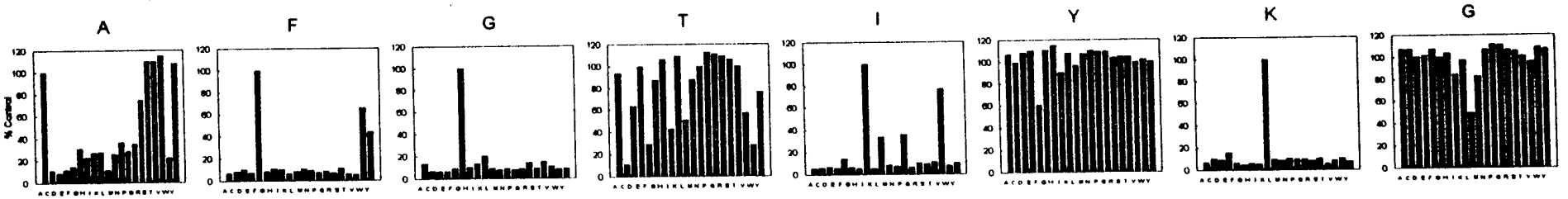
Figure 1.

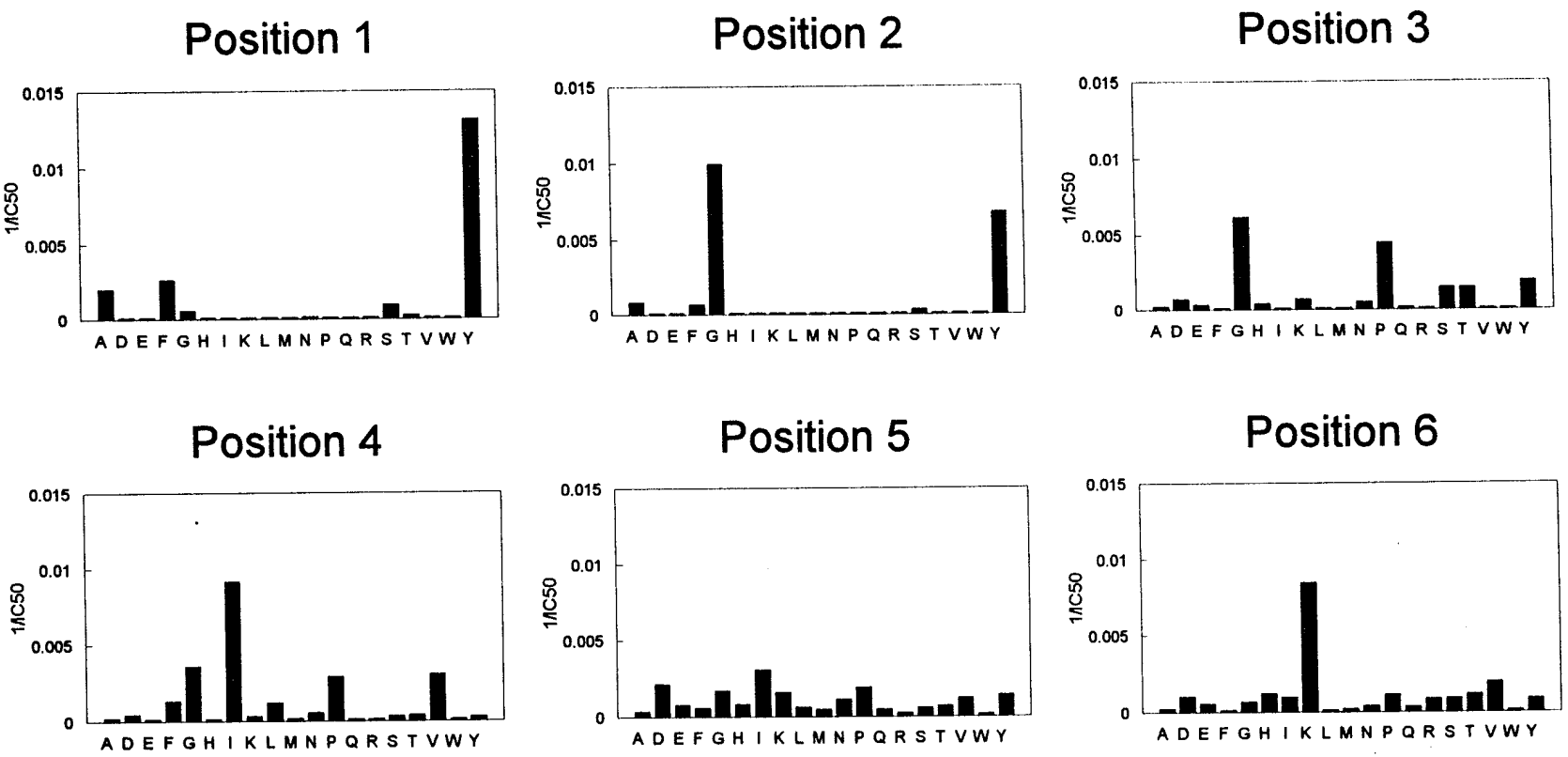
Direct ELISA (% Control)



Competitive ELISA (RPIF)

1.1	40.4	21.4	1.4	5.5	1.3	20.3	1.4

Figure 2.


Position 1
Position 2
Position 3
Position 4
Position 5
Position 6
1/IC50
0.015
0.01
0.005
0
A D E F G H I K L M N P Q R S T V W Y

igure 3.

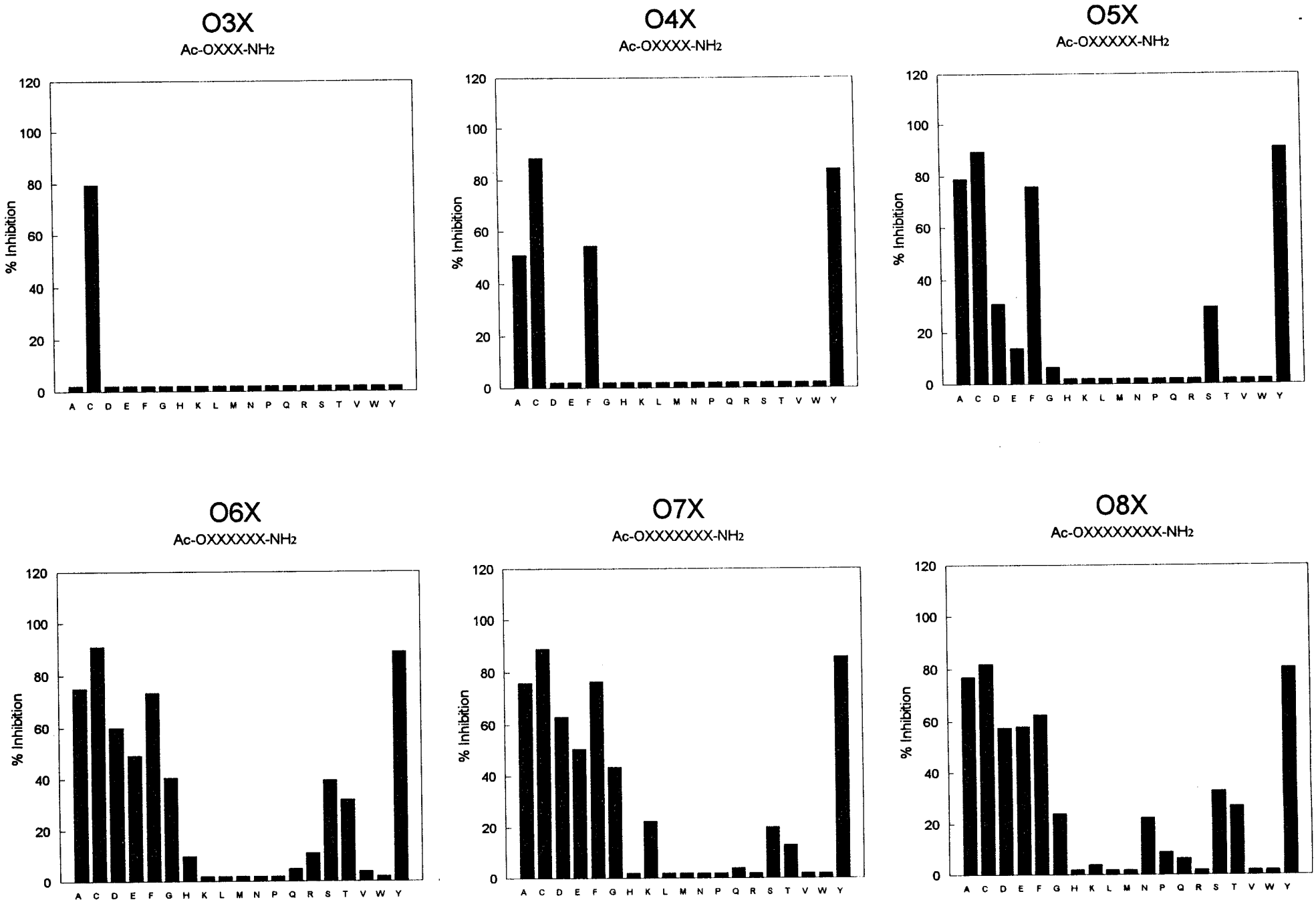

O3X
Ac-OXXX-NH2
O4X
Ac-OXXXX-NH2
O5X
Ac-OXXXXX-NH2
O6X
Ac-OXXXXXX-NH2
O7X
Ac-OXXXXXXX-NH2
O8X
Ac-OXXXXXXXX-NH2
% Inhibition
0
20
40
60
80
100
120
A C D E F G H K L M N P Q R S T V W Y

Figure 4.

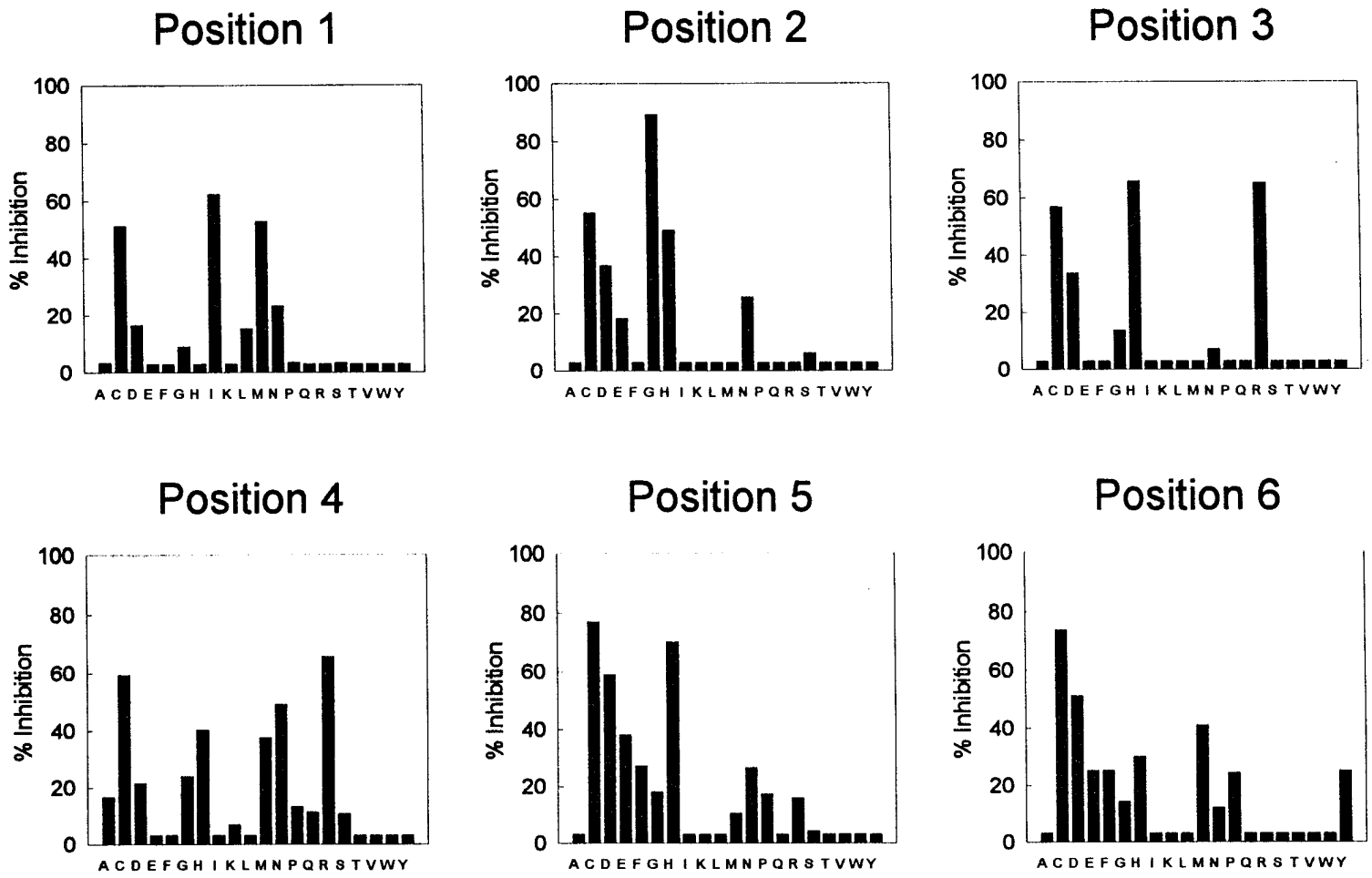
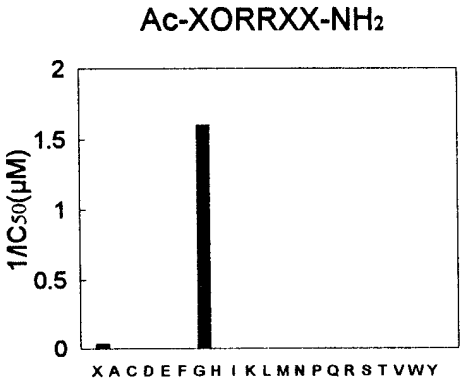
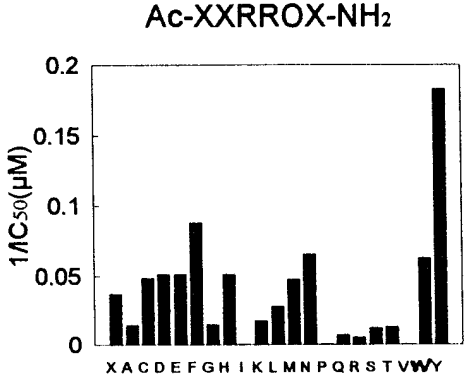
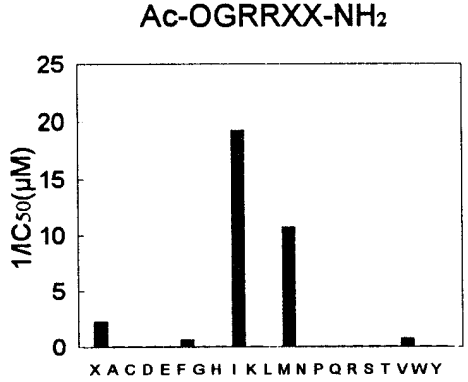

Position 1
Position 2
Position 3
Position 4
Position 5
Position 6
% Inhibition
0
20
40
60
80
100
A C D E F G H I K L M N P Q R S T V W Y

Figure 5.

Ac-XXO$_3$0$_4$XX-NH$_2$	IC$_{50}$(μM)
RR	131
WC	1200
CY	1211







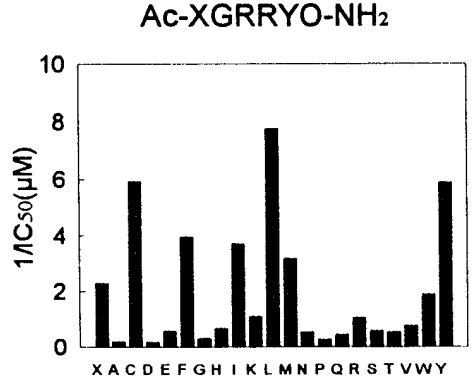


Table 1. IC_{50} values for individual peptides derived from hexapeptide PS-SCL.

		IC_{50}(μM)
1	Ac- Y G G I Y K -NH_2	60
2	Ac- Y G G I G K -NH_2	97
3	Ac- Y G G I N K -NH_2	32
4	Ac- Y G P I Y K -NH_2	2.7
5	Ac- Y G P I G K -NH_2	12.7
6	Ac- Y G T I N K -NH_2	2.4
7	Ac- Y G T I Y K -NH_2	19
8	Ac- Y G T I G K -NH_2	36
9	Ac- Y G T I N K -NH_2	10.3
10	Ac- Y G T I D K -NH_2	3.3
11	Ac- Y G G I D K -NH_2	15
12	Ac- Y G G I D G -NH_2	>1800
13	Ac- Y G G I I K -NH_2	105
14	Ac- Y G G I I G -NH_2	>1800
15	Ac- Y G G I K K -NH_2	120
16	Ac- Y G G I K G -NH_2	>1800
17	Ac- Y G G I P K -NH_2	75
18	Ac- Y G G I P G -NH_2	>1800
19	Ac- Y G K I D K -NH_2	6.2
20	Ac- Y G K I D G -NH_2	>1800
21	Ac- Y G K I I K -NH_2	27
22	Ac- Y G K I I G -NH_2	>1800
23	Ac- Y G K I K K -NH_2	32
24	Ac- Y G K I K G -NH_2	>1800
25	Ac- Y G K I P K -NH_2	28
26	Ac- Y G K I P G -NH_2	>1800
27	Ac- Y G P I D K -NH_2	1.2
28	Ac- Y G P I D G -NH_2	>1800
29	Ac- Y G P I I K -NH_2	5.5
30	Ac- Y G P I I G -NH_2	>1800
31	Ac- Y G P I K K -NH_2	6.1
32	Ac- Y G P I K G -NH_2	315
33	Ac- Y G P I P K -NH_2	4.1
34	Ac- Y G P I P G -NH_2	>1800
35	Ac- Y G S I D K -NH_2	5.9
36	Ac- Y G S I D G -NH_2	>1800
37	Ac- Y G S I I K -NH_2	23
38	Ac- Y G S I I G -NH_2	>1800
39	Ac- Y G S I K K -NH_2	32
40	Ac- Y G S I K G -NH_2	>1800
41	Ac- Y G S I P K -NH_2	16
42	Ac- Y G S I P G -NH_2	>1800
43	Ac- Y Y G I D K -NH_2	392
44	Ac- Y Y G I D G -NH_2	>1800
45	Ac- Y Y G I I K -NH_2	>1800
46	Ac- Y Y G I I G -NH_2	>1800
47	Ac- Y Y G I K K -NH_2	>1800
48	Ac- Y Y G I K G -NH_2	>1800
49	Ac- Y Y G I P K -NH_2	>1800
50	Ac- Y Y G I P G -NH_2	>1800
51	Ac- Y Y K I D K -NH_2	>1800
52	Ac- Y Y K I D G -NH_2	>1800
53	Ac- Y Y K I I K -NH_2	>1800
54	Ac- Y Y K I I G -NH_2	>1800
55	Ac- Y Y K I K K -NH_2	>1800
56	Ac- Y Y K I K G -NH_2	>1800
57	Ac- Y Y K I P K -NH_2	>1800
58	Ac- Y Y K I P G -NH_2	>1800
59	Ac- Y Y P I D K -NH_2	310
60	Ac- Y Y P I D G -NH_2	>1800
61	Ac- Y Y P I I K -NH_2	>1800
62	Ac- Y Y P I I G -NH_2	>1800
63	Ac- Y Y P I K K -NH_2	>1800
64	Ac- Y Y P I K G -NH_2	>1800
65	Ac- Y Y P I P K -NH_2	957
66	Ac- Y Y P I P G -NH_2	>1800
67	Ac- Y Y S I D K -NH_2	>1800
68	Ac- Y Y S I D G -NH_2	>1800
69	Ac- Y Y S I I K -NH_2	>1800
70	Ac- Y Y S I I G -NH_2	>1800
71	Ac- Y Y S I K K -NH_2	>1800
72	Ac- Y Y S I K G -NH_2	>1800
73	Ac- Y Y S I P K -NH_2	>1800
74	Ac- Y Y S I P G -NH_2	>1800

Table 2. IC_{50} values for most active mixtures from dual-defined PS-SCLs.

Ac-O_1O_2XXXX-NH_2	IC_{50}(μM)	Ac-XXO_3O_4XX-NH_2	IC_{50}(μM)	Ac-XXXXO_5O_6-NH_2	IC_{50}(μM)
YG	50	YG	58	DK	167
FG	89	FG	103	EK	239
YY	459	DI	361	AK	499
AY	639	GP	434	YK	571
FY	1172	GT	539	QK	728
AF	1796	PI	555	SK	730
		LI	564	KK	773
		GE	609	IK	796
		GQ	615		
		AI	636		

Table 3. IC_{50} values for individual peptides derived from hexapeptide PS-SCL.

	Peptides	IC_{50}(μM)
1	IGHHDD-NH_2	785.7
2	IGHHHD-NH_2	3500.0
3	IGHHRD-NH_2	653.7
4	IGHRDD-NH_2	68.0
5	IGHRHD-NH_2	70.0
6	IGHRRD-NH_2	4.6
7	IGRHDD-NH_2	30.3
8	IGRHHD-NH_2	54.3
9	IGRHRD-NH_2	5.9
10	IGRRDD-NH_2	2.4
11	IGRRHD-NH_2	2.5
12	IGRRRD-NH_2	5.3
13	MGHHDD-NH_2	1652.5
14	MGHHHD-NH_2	2978.0
15	MGHHRD-NH_2	200.3
16	MGHRDD-NH_2	50.3
17	MGHRHD-NH_2	44.7
18	MGHRRD-NH_2	2.9
19	MGRHDD-NH_2	52.0
20	MGRHHD-NH_2	5.7
21	MGRHRD-NH_2	1.8
22	MGRRDD-NH_2	1.6
23	MGRRHD-NH_2	0.6
24	MGRRRD-NH_2	2.6